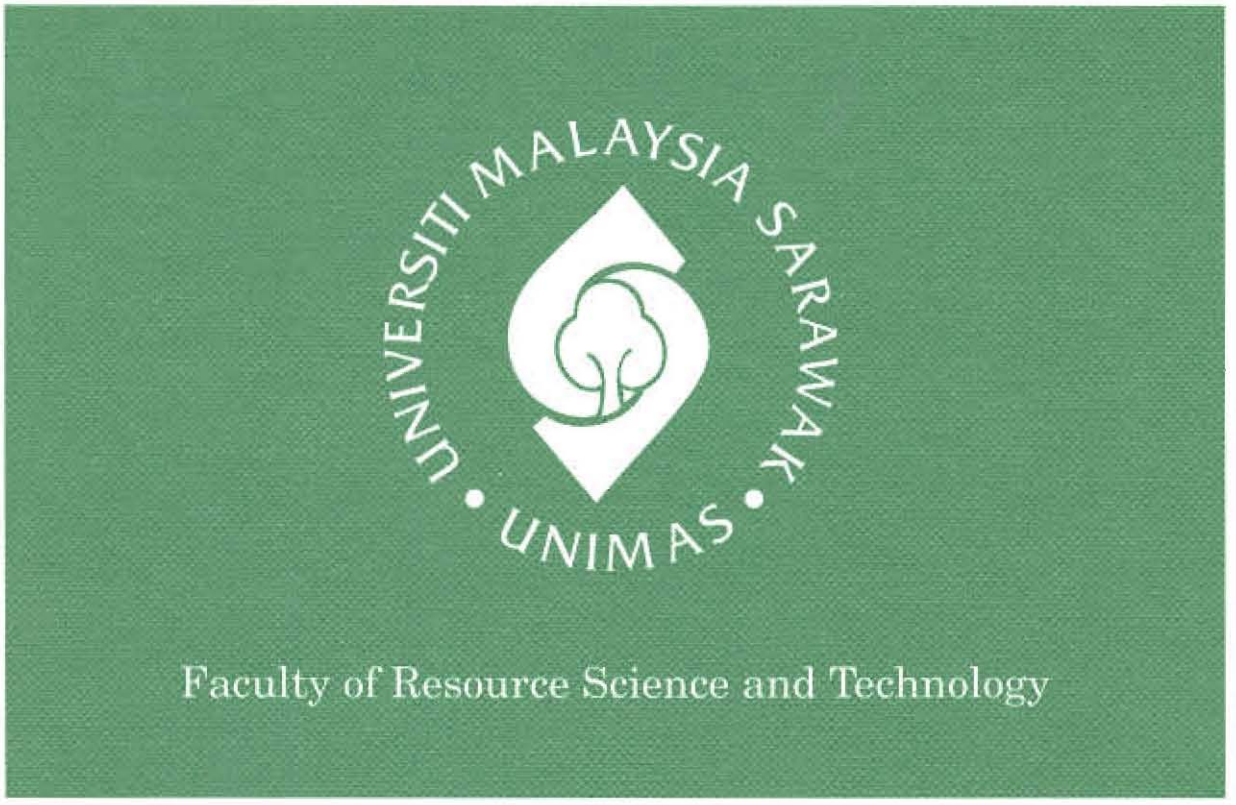

MOLECULAR CLONING AND CHARACTERIZATION OF GENE ENCODING GIBBERELLIN 2-OXIDASE (GA2OX) FROM *SHOREA PARVIFOLIA* DYER *PARVIFOLIA*

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This project is submitted in partial fulfilment of the requirements for the degree of Bachelor of Science with Honours (Resource Biotechnology)

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LIST OF ABBREVIATIONS

At	*Arabidopsis thaliana*
cDNA	Complemetary DNA
ClA	Chloroform-Isoamyl Alcohol
CPS	copalyl diphosphate synthase
CTAB	Cetyltrimethylammonium Bromide
dH_2O	Distilled water
DNA	Deoxyribonucleic Acid
DNase	Deoxyribonuclease
DEPC	Diethyl pyrocarbonate
dNTP	Deoxynucleotide triphosphate
EBl	European Bioinfomatics Institute
EDTA	Ethylenediamine tetraacetic acid
EtBr	Ethidium Bromide
GA	Gibberellin
GA2ox	Gibberellin 2 oxidase
GGDP	geranylgeranyl diphosphate
KAO	*ent*-kaurenoic acid oxidase
KO	*ent*-kaurene oxidase
Hypp	Hybrid poplar (*Populus tremula* X *Populus alba*)
KS	*ent*-kaurene synthase
IPTG	Isopropyl β thiogalactopyranoside
LB	Luria Bertani
$MgCl_2$	Magnesium Chloride
NaAc	Sodium acetate
NaCl	Sodium choride
NCBI	National Center for Biotechnology Information
PCI	Phenol: Chloroform: Isoamyl alcohol
PCR	Polymerase Chain Reaction
RACE	Rapid amplification of cDNA ends
RE	Restriction enzyme
RNA	Ribonucleic Acid
RNase	Ribonuclease
RT-PCR	Reverse Transcriptase-Polymerase Chain Reaction
Ta	Annealing temperature
Tm	Melting temperature
UV	Ultraviolet
X-GAL	5-bromo-4-chloro-3-indolyl-β-D-galactoside

ABSTRACT

Semidwarf tree varieties have provided many advantages to forestry as produce higher wood quality. The gene which is responsible for dwarfism varieties is the gibberellin 2-oxidase (GA2ox). The catabolic pathway of the gibberellin (GA) in plant which involve the GA2ox enzyme will catalyses the conversion of bioactive GAs compound into inactive form that cause dwarfism on plant. In this study, *Shorea parvifolia* Dyer *parvifolia* was chosen due to its commercial value and strong adaptability. The RNA was isolated from the species using the optimized RNA isolation protocol for *Shorea parvifolia* Dyer *parvifolia* from Plant Genomic Laboratory, Unimas. Two pairs of primers, namely At primer and Hypp primer were constructed based on the known GA2ox sequences obtained from gene bank databases. Reverse transcriptase-Polymerase Chain Reaction (RT-PCR) was performed and a DNA fragment of 515 bp from the PCR amplification with the At primer was purified. The DNA fragment was cloned into pGEMT®-Easy vector and sent for sequencing. However, the sequence result showed low homology to *Bacillus anthracis*, *Homo sapiens* chromosome 17, Human BAC clone CTA-208O3, Uncultured bacterium clone YC01A01, and *Helicobacter pylori* of unknown function instead of GA2ox.

Keywords: gibberellin (GA), gibberellin 2-oxidase (GA2ox), *Shorea parvifolia* Dyer *parvifolia*, RT-PCR, DNA

ABSTRAK

Pokok kerdil membawa banyak kebaikan kepada bidang perhutanan seperti menghasilkan kayu yang berkualiti. Gen yang bertanggungjawab bagi pelbagai kekerdilan adalah gibberellin 2-oxidase (GA2ox). Proses katabolik gibberellin (GA) pada tumbuhan melibatkan enzim GA2ox akan bertindak balas untuk menukarkan GA aktif kepada bentuk yang tidak aktif yang akan menyebabkan kekedilan pada tumbuhan. Dalam kajian ini, Shorea parvifolia Dyer parvifolia telah dipilih disebabkan nilai komersialnya dan kebolehan adaptasi. RNA telah dipencilkan dari spesies menggunakan protokol pemencilan RNA dari Makmal Genomik Tumbuhan, Unimas. Dua pasang pencetus, iaitu pencetus At dan pencetus Hypp telah direka berasakan jujukan GA2ox daripada pangkalan data gen bank. Tindak Balas Berantai Polimerase-Reverse transcriptase (RT-PCR) telah dijalankan dan satu serpihan DNA bersaiz 515 bp daripada amplifikasi PCR dengan pencetus At telah ditulenkan. Serpihan DNA telah diklonkan ke vektor pGEMT®-Easy dan dihantar untuk proses penjujukan DNA. Walau bagaimanapun, keputusan jujukan yang diperolehi menunjukkan homologi yang rendah kepada Bacillus anthracis, Homo sapiens chromosome 17, Human BAC clone CTA-208O3, uncultured bacterium clone YC01A01 dan Helicobacter pylori dengan fungsi yang belum diketahui tetapi bukan GA2ox.

Kata Kunci: gibberellin (GA), gibberellin 2-oxidase (GA2ox), Shorea parvifolia Dyer parvifolia, RT-PCR, DNA

CHAPTER I

INTRODUCTION

Shorea is one of the most important economical timber species in tropical Asia. It is grouped under the family of Dipterocarpaceae. Among the 194 of *Shorea* species, there are 163 species occur in Malaysia. *Shorea* is divided into two groups, which are lightweight hard wood (light red meranti) and lightweight to medium-heavy hardwood (dark red meranti). *Shorea parvifolia* which is locally known as meranti sarang punai or light-red meranti, meranti samak (Sarawak), is grouped under lightweight hardwood (Soerianegara & Lemmens, 1994).

Gibberellin (GA) is one of the important growth regulators in plants which have important role in plant growth and developmental processes (Davies, 1995). Besides gibberellin, there are other plant growth regulators, such as auxin, cytokinin, ethylene and abscisic acid which also have important role in plant development but gibberellin has the unique ability to promote extensive growth in many plant species (Salisbury & Ross, 1992). The levels of bioactive GAs are controlled by both GA biosynthetic and catabolic pathways in which the pathway has three stages (Olszewski et al., 2002).

According to Ross et al. (1995), Gibberellin 2-oxidase (GA2ox) is the multifunctional enzyme which involved in GA catabolic pathway to catalyse the conversion of bioactive GAs compound into inactive form. Thus, GA 2-oxidase has an important role in controlling the plants growth by reducing the bioactive GAs level. GA 2-oxidase gene expression is activated by biologically active GA or GA precursors, whereas GA 20-oxidase and GA 3β-

hydroxylase (both enzymes are involved in the GA biosynthetic pathway) are inactivated by bioactive GA (Thomas et al., 1999). Therefore, an effective feed-forward and feed-back regulation can be maintained, respectively for the two different pathways (Thomas et al., 1999).

Gibberellin 2-oxidase (GA2ox) gene has been identified in playing an important role in the regulation of the elongation growth in the garden pea (*Pisum sativum*) (Lester et al., 1999; Martin et al., 1999) and rice *Oryza sativa* (Sakamoto et al., 2001). GA2ox has been successfully isolated from a few plant species, such as runner bean (*Phaseolus coccineus*) (Thomas et al., 1999), *Arabidopsis* (Thomas et al., 1999; Schomburg et al., 2003), garden pea (*Pisum sativum*) (Lester et al., 1999; Martin et al., 1999) and rice (*Oryza sativa*) (Sakamoto et al., 2001).

Schomburg et al. (2003) stated that GA2ox genes may provide advantages in agriculture to control plant stature without the use of chemical treatments to inhibit GA biosynthesis because deficiencies in GAs will cause dwarfism in plant. Nowadays, there are many inhibitors of GA biosynthesis are commercially used as retardants such as chloroethyltrimethylammonium chloride which is used on cereal field to decrease the growth of the stems in order to enhance the strength of cereal stems (Heldt, 1997). However, plant growth retardants that alter the GA biosynthesis of the plant stature require continuous applying the synthetic chemicals which are expensive, and can affect the environmental conditions (Busov et al., 2003).

Bradshaw and Strauss (2001) also stated that semidwarf has also provided advantages to forestry. Dwarf or semidwarf varieties provide many benefits to human being as it is easy to manage and maintain. Semidwarf trees may have higher biomass productivity because of

the reduced investment in root mass, they may produce higher quality wood due to reduced bending and leaning (reaction wood), and they are also more resistant to be damaged by rain and wind (Busov et al., 2003). Besides that, if the trees are large in size and tall, they required intensive pruning to avoid damage to the surrounding. Pruning and removal of the trees also required high costs (Busov et al., 2003). As mentioned by Busov et al. (2003), reducing in the height of the tree species also could reduce the propensity of the tree progeny to spread in wild populations that will eventually produce exotic tree species. The exotic tree species sometimes can also cause disruption in our ecology (Richardson, 1998). Since GA2ox gene is important in agriculture, it is essential to obtained more information on this gene.

Until recently, not many studies have been done on the GA2ox on forest trees species. Most of the studies of the GA2ox gene are on non-woody species, such as runner bean, garden pea and rice. Although some studies of GA2ox gene have been carried out on poplar tree (Busov et al., 2003), the GA2ox gene of other trees is still remains poorly understood. Thus, the objective of the study is to isolate the gene encoding gibberellin 2-oxidase (GA2ox) from *Shorea parvifolia* Dyer *parvifolia* using Reverse Transcriptase-Polymerace Chain Reaction (RT-PCR).

CHAPTER II

LITERATURE REVIEW

2.1 *Shorea spp.*

Shorea species consists of 194 species distributed in Sri Langka, India, Indo-China and Malaysia, in which 163 species occur in Malaysia. The greatest diversity are distributed in Borneo (62 species), followed by Sumatera (23 species), Peninsular Malaysia (19 species), the Philippines (5 species) and the Moluccas (1 species). However, red meranti is not found in Sulawesi (Soerianegara & Lemmens, 1994).

Shorea is divided into two groups based on the property of the hardwood. Lightweight hardwood is known as light red meranti, the species in this group include *Shorea leprosula*, *Shorea parvifolia* and *Shorea smithiana*. Lightweight to medium-heavy hardwood is known as dark red meranti. The species in this group include *Shorea curtisii*, *Shorea macrantha*, *Shorea ovata*, *Shorea paucifolia* and *Shorea platyclados* (Soerianegara & Lemmens, 1994).

Shorea could grow up to 70 m tall and up to 255 cm in diameter, with bold stem for 10-42 m and extensive branches at the terminal shoot (Soerianegara & Lemmens, 1994). *Shorea* leaves are usually glabrous, occasionally boat-shaped, not plicate, rarely peltate, and scalariform in tertiary venation (Newman et al., 1996).

Shorea flowers are secund or distichous, scented, bisexual, rather crowded, and inflorescences at the apical shoots or axillary (Soerianegara & Lemmens, 1994). *Shorea*

flower usually contains 15 to 70 stamens, and each anther constitute of 4 pollen sacs (Newman et al., 1996). The nuts contained only 1 seed, subglobose to ovate in shape and free from calyx. The seedling is of epigeal germination, the pericarp splitting irregularly, the young leaves are arranged spirally and often larger than the leaves of mature tree (Soerianegara & Lemmens, 1994).

Due to its non-siliceous nature, red meranti is the commonest utility for construction in western Malaysia. The general use of the wood is for plywood and veneer. However, the wood is not suitable to use in contact with ground or in exposed conditions, unless properly treated, because the wood is not durable and rapidly attack by fungi and insects (Soerianegara & Lemmens, 1994).

2.2 *Shorea parvifolia* Dyer *parvifolia*

Shorea parvifolia Dyer *parvifolia* (Figure 2.1) is distributed around Peninsular Thailand, Peninsular Malaysia, Sumatra and Borneo (Newman et al., 1996). According to Soerianegara and Lemmens (1994), *Shorea parvifolia* Dyer *parvifolia* is locally known as meranti sarang punai.or light-red meranti, meranti samak (Sarawak) and saraya punai (Sabah). At east Kalimantan, it is known as abang gunung and kontoi burung or Tengkawang at west Kalimantan but it is known as saya-luang in Thailand.

Figure 2.1 *Shorea parvifolia* Dyer *parvifolia*

Shorea parvifolia Dyer *parvifolia* is tall in which it can grow until 65 m tall with bole branchless for 18-30 m and up to 190 cm diameter, buttresses up to 4 m high (Kimura and Nishiyama, 1999). *S. parvifolia* Dyer *parvifolia* leaves (Figure 2.2) are generally ovate, rather small (5-9 cm long), and smooth. Stipules are generally elliptical, ovate, acute or obtuse. The flowers (Figure 2.3) are small with white petals, tinged pink at the base. There are 15 stamens in each flower (Newman et al., 1996).

(a) (b)

Figure 2.2: *Shorea parvifolia* Dyer *parvifolia*, (a) adaxial and (b) abaxial surface of the leaf

Figure 2.3: Flowers of *Shorea parvifolia* Dyer *parvifolia* (Adapted from Kimura and Nishiyama, 1999).

2.3 Gibberellin

Gibberellin (GA) is one of the plant growth regulators. GAs involve in plant developmental processes such as seed germination, stem elongation, leaf expansion, trichome development, fruit development and flowering (Davies, 1995). GAs are tetracyclic diterpenoid acids, which

exist in angiosperms, gymnosperms, ferns, and probably also in mosses, alga, and some fungi and it can be found at immature seeds, young leaves, and roots (Salisbury & Ross, 1992).

According to Olszewski et al. (2002), the gibberellin biosynthetic pathway in plants can be divided into 3 stages (Figure 2.4). The first stage involved in the biosynthesis of *ent*-kaurene from geranylgeranyl diphosphate (GGDP) by copalyl diphosphate synthase (CPS) and *ent*-kaurene synthase (KS) in proplastids. As for the second stage, *ent*-kaurene is converted to *ent*-kaurenoic acid by *ent*-kaurene oxidase (KO) and lastly forms GA_{12} by *ent*-kaurenoic acid oxidase (KAO) through microsomal cytochrome P450 monooxygenases. GA_{12} can also be further converted to GA_{53} by 13-hydroxylation. At the final stage, GA_{12} and GA_{53} can be modified to become various GA intermediates and bioactive GAs, such as GA_1 and GA_4, by oxidative reaction which involves 2-oxoglutarate-dependent dioxygenases, GA 20-oxidase (GA20ox) and GA 3-oxidase. Inactive catabolic can also be formed from bioactive GAs by 2β-hydroxylation, GA 2-oxidase (GA2ox). All the reactions in stage three take place in the cytoplasm.

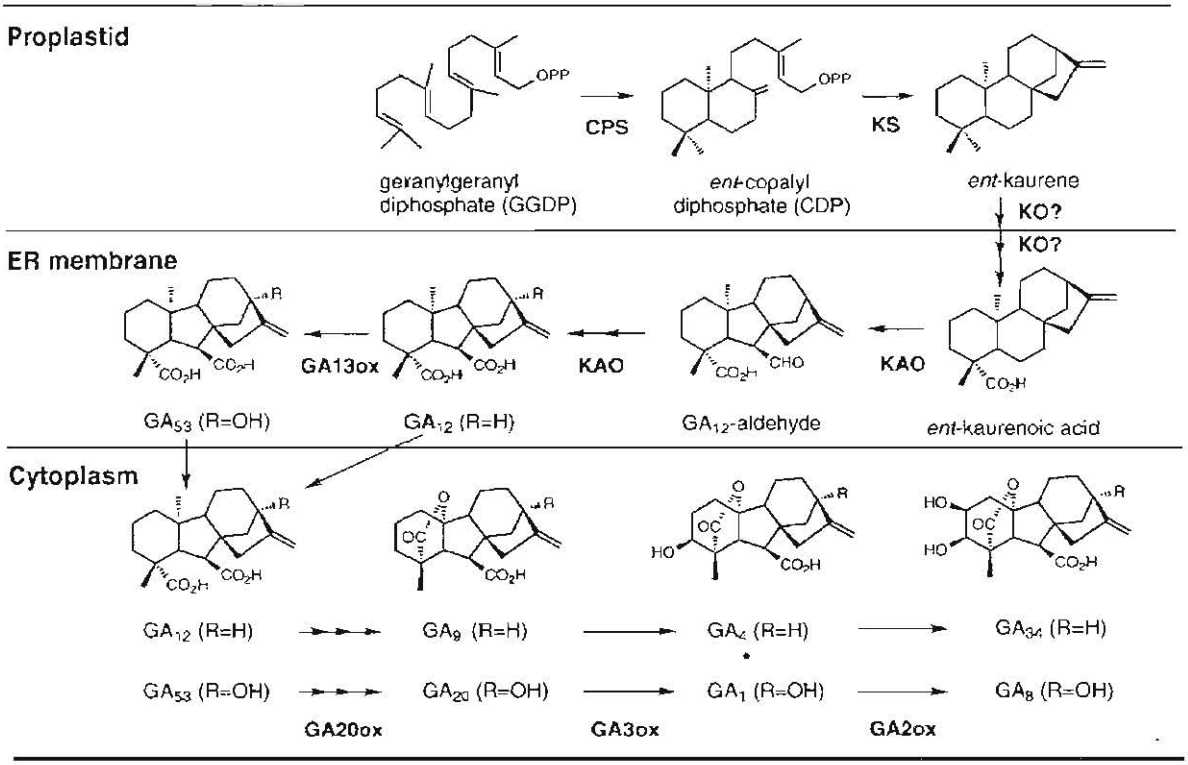

Figure 2.4: Major GA biosynthetic and catabolic pathway in higher plant. GA_4 and GA_1 are the bioactive GAs, and GA_{34} and GA_8 are their inactive catabolites (Adapted from Olszewski et al., 2002).

One hundred and twenty six GAs have been identified to date in plants, fungi and bacteria (http://www.plant-hormones.info/gibberellins.htm). However, most of the GAs are intermediates or by-products (Heldt, 1997). Examples of bioactive GAs synthesized by higher plants are GA_1, GA_3, GA_4 and GA_7, whereas inactive GAs are GA_4, GA_{29}, GA_{38} and GA_{51} (Schomburg et al., 2003).

2.4 Gibberellin 2-oxidase (GA2ox)

Gibberellin 2-oxidase (GA2ox) is a multifunctional catabolic enzyme, which catalyses the conversion of active GAs compound into inactive form (Ross et al., 1995). Initially GA 2-oxidase coverts GA_9 to GA_{51} through 2β-hydroxylation, the GA_{51} is then oxidise at C-2 to give rise to GA_{51}-catabolite, which is the inactive products that cannot be converted to active form (Thomas et al., 1999). GA 2-oxidase gene expression is activated by GA, whereas GA 20-oxidase and GA 3β-hydroxylase genes are inactivated by bioactive GA (Thomas et al., 1999). Therefore, providing an effective feed-forward and feed-backward regulation, respectively for the two different pathways (Thomas et al., 1999).

GA2ox gene of garden pea (*Pisum sativum*) showed that the gene plays an important role in the regulation of elongation growth of the garden pea (Lester et al., 1999; Martin et al., 1999). Besides that, *Oryza sativa* GA 2-oxidase 1 (*Os*GA2ox1) that encodes GA2ox control the level of bioactive GAs in the shoot apex, in which the transgenic rice with over-expression of *Os*GA2ox1 cDNA inhibited stem elongation (Sakamoto et al., 2001). Schomburg et al. (2003) showed that the two GA 2-oxidase (*At*GA2ox7 and *At*GA2ox8) identified in *Arabidopsis* were required for normal elongation growth in tobacco because over-expression of both genes in tobacco caused dwarfing. Thus, GA 2-oxidase has played an important role in controlling the plants growth by reducing the bioactive GAs level.

Deficiencies in GAs will cause dwarfism in plant. Dwarf or semidwarf varieties have provided many benefits to human being as it is easy to mange and maintain. For example, in fruit trees, dwarf and semidwarf trees are more preferred by the farmers because it increased the efficiency of fruit collection, and easy to apply pesticide to the trees (Webster, 2002).

Recently, semidwarf varieties have been applied to poplar trees (Busov et al., 2003) as semidwarf also provides advantages to forestry (Bradshaw & Strauss, 2001).

The study carried out by Thomas et al. (1999) had successfully isolated and found at least two of the GA 2-oxidase genes that showed similar pattern of expression and abundance in growing tissues, in contrast to the 20-oxidase and 3β-hydroxylase which showed tissue-specific expression (Thomas et al., 1999). Other finding was GA-stimulated up-regulation of 2-oxidases, indicating there is a feed-forward regulation (Thomas et al., 1999).

Gibberellin 2-oxidase was also successfully isolated and cloned from the runner bean (*Phaseolus coccineus*) and three GA2ox cDNA were cloned from *Arabidopsis* (Thomas et al., 1999). GA2ox genes from transgenic hybrid poplar (*Populus tremula* x *Populus alba*) (Busov et al., 2003), garden pea (*Pisum sativum*) (Lester et al., 1999; Martin et al., 1999), rice (*Oryza sativa*) (Sakamoto et al., 2001), and *Arabidopsis* (Schomburg et al., 2003) were also isolated using reverse transcription-Polymerase Chain Reaction (RT-PCR).

2.5 Reverse-transcriptase Polymerase Chain Reaction (RT-PCR)

Reverse-transcriptase Polymerase Chain Reaction (RT-PCR) has becoming one of the widely used techniques in variety researches. According to Brown (2001), RT-PCR is a technique in which RNA is used as a template for polymerase chain reaction (PCR). RNA molecule is firstly converted to single-stranded cDNA with reverse transcriptase. After that, normal PCR method will be used to amplify the DNA in short period. RT-PCR is an extraordinary sensitive method used to detect low-abundance mRNA (O'Connell, 2002), as few as 1-100 copies of specific mRNA (Shuldiner et al., 1993). The major shortcoming of this technique is

that false positives caused by contamination with the minutes qualities of DNA (Shuldiner et al., 1993).

CHAPTER III

MATERIALS AND METHODS

3.1 RNA extraction and purification

3.1.1 Plant materials

Inner bark tissue of *S. parvifolia* Dyer *parvifolia* mother tree was collected from Semengoh Forest Reserve, Semongoh. The inner bark of *S. parvifolia* Dyer *parvifolia* was pealed from the tree and subsequently a scrapper was used to scrap out the inner bark tissues. Then, the inner bark tissues were kept in a small plastic bag and sealed properly. After that, the inner bark tissues were frozen immediately in the liquid nitrogen and subsequently stored in the freezer at -80°C in order to minimize tissue damage and degradation.

3.1.2 RNA isolation

The total RNA was isolated according to the optimized RNA isolation protocol for *S. parvifolia* Dyer *parvifolia* (Lau & Ho, 2006). Cetyltrimethylammonium bromide (CTAB) buffer was firstly preheated at 65°C for an hour. After that, the inner bark sample of *S. parvifolia* Dyer *parvifolia* was ground with liquid nitrogen. Later, the sample was added to the preheated CTAB buffer and incubated at 65°C for half an hour. Subsequently, the supernatant was transferred to another Falcon tube. An equal volume of chloroform was added to the supernatant and centrifuged at 14,000 rpm for 10 minutes. This step was